SYNOPSIS

Study of Molecular Mechanism of Gall Development in Medicinally Important Plants Using Omics Approach

By

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1. Background

Pistacia chinensis subsp. *integerrima* (J. Stewart) Rech. f. is a small deciduous tree native to Asia, particularly in Pakistan, India, and Iran (Bibi *et al*, 2015; Ahmad *et al*, 2020). This tree is known for its medicinal properties and commonly used in traditional medicine to treat various ailments, such as diarrhea, dysentery, and respiratory infections. One interesting aspect of *P. integerrima* is the formation of galls on its leaves (Bibi *et al*, 2015). Galls are abnormal growths that occur on plants in response to insect or mite infestations, as well as bacteria, fungi, or viruses. In the case of *P. integerrima*, galls appear to be formed by insects. The galls on *P. integerrima* are spherical or oval-shaped and range in size from 10 to 20 millimeters. They are green at first, but later turn red or brown as they mature. Aphids are frequently discovered within galls. The insect's saliva is believed to contribute to the plant's growth around the gall, providing a protective barrier (Takeda *et al*, 2021). The galls of *P. integerrima* are also used in traditional medicine. They are astringent and are used to treat diarrhea, dysentery, and fever. The galls are usually dried, powdered, and mixed with other herbs to make a therapeutic formulation (Uddin *et al*, 2011; Kaur and Singh, 2015; Warrier *et al*, 1995; Rauf *et al*, 2015).

P. integerrima holds a prominent place in several essential polyherbal Ayurvedic formulations like dasamularista, chayavanaprasa, shringyadi leha, and shringyadi churna. This plant exhibits potential pharmacological properties encompassing antibacterial, antioxidant, anti-inflammatory, cardioprotective, anticancer, antidiarrheal, anticonvulsant, and muscle relaxant effects. In Sanskrit, *P. integerrima* is widely recognized as "Karkatshringi." *P. integerrima* possesses Ayurvedic properties characterized by its Virya (potency) being "Ushna" or hot, with a Vipak (metabolic property) of "Katu" or pungent. It exhibits the Guna (physical properties) of being "Laghu" or light and "Ruksha" or dry. In terms of Rasa (taste), it encompasses "Katu" or pungent and "Tikta" or bitter flavors. It is known to harmonize the Kapha and Vata doshas within the body (Grover M, 2021).

The process of gall formation is complex and involves various interactions between the host plant and the invading organism (Nabity *et al*, 2013). The formation of galls begins when the pest insect or mite lays eggs on the plant's surface or injects saliva or other chemicals into the plant's tissues. These substances act as a trigger and stimulate the plant cells to divide and form a protective barrier around the site of invasion. This barrier is called a gall and provides

a safe and nutrient-rich environment for the pest organism to feed and reproduce (Giron *et al*, 2016). One of the most important changes that occur during gall formation is the redirection of the plant's nutrient and energy flow to the site of invasion. The pest organism is able to manipulate the plant's hormone signaling pathways, causing the production of compounds that promote the growth and development of the gall. The pest organism can also manipulate the plant's defense response, allowing it to evade or suppress the plant's immune system (Stone *et al*, 2003).

The exact molecular mechanism of plant gall formation is still unknown. To conserve the galls of this plant, it is necessary to understand the molecular mechanism of gall formation. So in this study, we made an attempt to study the molecular mechanism of gall formation through a different omics approach such as genomics, transcriptomics, metabolomics and microbiome. We used an integrative strategy that incorporates multi-omics data to emphasize the interrelationships of the implicated biomolecules and their activities in order to understand complicated biological processes holistically. Using these omics techniques we studied gall formation at molecular level such as the expression of genes in gall, the enriched biochemical pathways in gall, the community of microbes associated with gall formation, different chemical compounds (metabolites) which are involved in gall formation etc.

The demand for *P. integerrima* for therapeutic usage is high, but the available population of the trees don't meet the demand. The study on the molecular mechanism of gall formation is important. Also, Ayurveda mentions the use of galls of *T. chebula* as substitute to *P. integerrima.* So in this study an attempt was made to capture the metabolites of both the plant galls.

2. Aim and Objectives

Aim:

To understand the molecular mechanism behind the formation of plant galls

Objectives:

1. To determine the function of genes and the elements that regulates the gall formation in *P. integerrima* using whole genome and transcriptome sequencing

2. To identify the potential microbes associated with gall in comparison to leaves of *P. integerrima*

3. To study the metabolite diversity in galls and leaves of *P. integerrima* and *T. chebula*

3. Overview of Methodology

The samples of gall and leaf of *P. integerrima* were collected from its natural habitat. The entire genetic code of *P. integerrima*, as well as its leaf transcriptome, were analyzed through the use of Illumina sequencing technology to comprehend the plant's biological traits and acquire a genetic understanding of how its natural compounds are biosynthesized. The genome assembly was functionally annotated, and gene prediction was accomplished through an integrated genome annotation workflow. Pathway analysis was executed utilizing the KEGG database. The RNASeq study was conducted for both gall and leaf from *P. integerrima* to identify differentially expressed genes between gall and leaf tissues. Through microbiome study, we identified potential microbes (both fungi as well as bacteria) associated with gall and leaf of *P. integerrima* using 16S rRNA and ITS sequencing. Also the putative bacterial genes and their respective biological pathways were identified from both gall and leaf. The metabolite profiling of gall and leaf of *P. integerrima* and *T. chebula* were identified using a mass spectrometry-based global metabolomics method.

Further, correlation analysis of data generated from different omics study was carried out. The correlation between microbes and metabolites was determined to identify potential metabolites from microbes in both gall and leaf.

4. Results

The genome analysis of *P. integerrima* resulted in a draft genome assembly of 462 Mb with N50 16,145 bp. Within this assembly, a total of 39,452 genes were discovered, with 18,492 of these genes containing RNA or protein evidence. The genes responsible for the biosynthesis of several plant secondary metabolites, such as flavonoids and terpenoids, were characterized. Additionally, noncoding RNA miR397 and miR828 families were identified, which primarily target the laccase (LCA) and MYB protein, respectively. Phylogenetic analysis demonstrated

that *P. integerrima* is more genetically similar to P. vera. The Transcriptome analysis yielded 73,572 transcripts for gall and 46,571 transcripts for leaf. Total 17,257 genes that were identified as differentially expressed in both gall and leaf. Genes responsible for phytohormone signaling pathways exhibited higher expression levels in gall when compared to leaf. Furthermore, we discovered 62 transcripts encoding serine/threonine protein kinase, which are associated with plant-aphid interactions.

From this study, the microbes that are known to have a direct role in plant gall formation couldn't be identified. However, certain bacterial taxa were identified, such as Aeromonas (with an abundance of 28% in gall and 20% in leaf), Pseudomonas (with an abundance of 2.1% in gall and 4% in leaf), and Bacillus (with an abundance of 3.2% and 8.5% in gall and leaf, respectively), which can have beneficial effects on plant growth and development. These bacteria promote nutrient acquisition, stimulate plant defenses, and suppress pathogenic microbes. These beneficial bacteria are often referred to as plant growth-promoting bacteria (PGPB) and can potentially contribute to gall formation in plants. The *Glomerellaceae* (96%) family was identified as the most abundant fungal family in gall.

Total 4414 non-redundant metabolites were identified from both gall and leaf of *P. integerrima*. In total, 981 metabolites were only found in the gall, while 1591 metabolites were only found in the leaf sample. Both gall and leaf had the remaining 1898 metabolites. In the case of *T. chebula,* 1252 metabolites were unique to gall whereas 1040 metabolites were unique to leaf samples. The remaining 1498 metabolites were identified in both gall and leaf. A total of 1593 metabolites were found to be shared between *P. integerrima* and *T. chebula* galls, while 1157 metabolites were unique to *T. chebula* gall and 1286 metabolites were exclusive to *P. integerrima* gall. Likewise, 1577 metabolites were identified to be common between *P. integerrima* and *T. chebula* leaves, with 961 metabolites being specific to *T. chebula* leaf and 1856 metabolites being unique to *P. integerrima* leaf. The gall of *P. integerrima* demonstrated enriched pathways, such as linoleic acid metabolism, taurine and hypotaurine metabolism, sesquiterpenoid and triterpenoid biosynthesis, and Sphingolipid metabolism, among others. Conversely, the leaf of *P. integerrima* showed enrichment in pathways such as taurine and hypotaurine metabolism, linoleic acid metabolism, indole alkaloid biosynthesis, and sulfur metabolism, among others. Various metabolites were discovered from distinct categories in both gall and leaf tissues, including prenol lipids,

organooxygen compounds, fatty acyls, carboxylic acids and derivatives, steroids and steroid derivatives, flavonoids, phenol ethers, tannins, and others.

From the correlation analysis between microbes and metabolites, it was observed that out of 40 bacterial genera (which were highly abundant in gall), 18 genera were linked to 286 metabolites and 77 metabolic pathways. Out of 286 metabolites associated with microbes, 13 metabolites were identified in gall from LC_MS/MS metabolite profiling. The 13 metabolites are Glyoxylic acid, Oxidized glutathione, Agmatine, Glyceric acid, N-Carbamoylputrescine, Betaine aldehyde, Serotonin, 7,8-Dihydropteroic acid, Fucose 1-phosphate, Coproporphyrinogen III, Dihydroneopterin triphosphate, 3-Mercaptolactic acid, P1,P4-Bis(5'-uridyl) tetraphosphate.

5. Conclusion

The comprehensive analysis of *P. integerrima* in this study has provided valuable insights into its genome, transcriptome, microbial communities, and metabolic pathways associated with gall formation. The draft genome assembly and gene discovery shed light on the genetic makeup of *P. integerima*, revealing the presence of key genes involved in the biosynthesis of secondary metabolites, including flavonoids and terpenoids. The transcriptome analysis identified differentially expressed genes between gall and leaf tissues, with a particular emphasis on phytohormone signaling pathways *viz.* auxin, cytokinins, brassinosteroid, gibberellins, abscisic acid, etc. These findings suggest that phytohormones play a crucial role in gall formation and provide avenues for further investigation into the regulatory mechanisms involved in this process.

Although the specific microorganisms responsible for gall formation could not be identified in this study, the presence of certain bacterial taxa, such as *Aeromonas, Pseudomonas*, and *Bacillus*, implies their potential involvement in promoting plant growth, nutrient acquisition, and suppressing pathogenic microorganisms. These beneficial bacteria, known as plant growth-promoting bacteria (PGPB), represent promising candidates for future research exploring their direct role in gall formation. Moreover, the dominance of the *Glomerellaceae* fungal family in gall highlights the significance of fungal communities in the gall microenvironment.

The metabolite analysis provided insights into the distinct metabolic profiles of gall and leaf tissues, revealing enriched pathways and the presence of several unique metabolites in each tissue type. Ayurveda suggests *T. chebula* galls as substitute for P. integerrima galls. Therefore a comprehensive metabolite profiling of *T. chebula* has been undertaken to elucidate the shared metabolite composition between *T. chebula* and *P. integerrima*. The identification of shared and unique metabolites between *P. integerrima* and *T. chebula* galls and leaves expands our knowledge of the biochemical composition and potential physiological functions of these plant species.

Overall, this study serves as a foundation for future investigations aiming to unravel the complex interactions between microbes, metabolites, and host plants during gall formation. The findings contribute to our understanding of the potential roles of beneficial microbial communities including bacteria and fungi and metabolic pathways in the development of galls in *P. integerrima*. The application of the results of this study holds promising prospects for applications in traditional medicine, plant health management and the exploration of novel bioactive compounds derived from gall-associated microbial and plant interactions.

6. References

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7. Publications

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2. De novo Transcriptome Analysis of Gall and Leaf of *Pistacia chinensis* subsp. *integerrima* (J. L. Stewart ex Brandis) Rech. f. (Communicated; Under peer review)